

PROTHROMBIN

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BY

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By prothrombin is meant the substance contained in blood-plasma and also, as direct experiments show, in blood-platelets, which in the presence of calcium salts is converted to active thrombin. There is a difference of opinion in regard to the factors concerned in this activation. Since calcium salts are present in blood-plasma, together with the prothrombin, and yet, under normal conditions, clotting does not occur, it would appear that either some third factor is necessary for the activation or else the effect of the calcium on the prothrombin is prevented by the presence of a protective substance. Fuld and Spiro,¹ and Morawitz,² have adopted the first alternative and have assumed that the third factor is a kinase (thrombokinase of Morawitz, cytozym of Fuld and Spiro), which does not exist in circulating blood but is furnished after shedding by some of the corpuscular elements (platelets). Thrombokinase may be obtained likewise from the tissue-cells in general. Bordet³ also assumes that the disintegrating platelets furnish an enzyme (cytozyme) that takes part in the formation of the thrombin, but the substance acted upon (serozyme) is not supposed to be present in the circulating blood. The present author has furnished evidence in several researches in support of the second of the alternatives mentioned above. It has been shown that an anti-thrombin exists in circulating blood in quantities sufficient to bind the prothrombin and thus prevent its activation by the

¹ Fuld and Spiro: Hofmeister's Beiträge, 1904, v, 174.

² Morawitz: Hofmeister's Beiträge, 1903, iv, 381, and Deutsches Archiv für klin. medicin, 1904, lxxix, 1 and 215.

³ Bordet and Delange: Annals de l'Institut Pasteur, 1912, xxvi, 657 and 737.

calcium. Normal circulating plasma is in fact an incoagulable or slowly coagulable liquid, owing to the excess of antithrombin. When blood is shed the platelets in disintegrating furnish a material that neutralizes the antithrombin and permits the calcium to activate the prothrombin. This substance derived from the platelets and furnished also by the other corpuscles of the blood (Austin and Pepper⁴) or the tissue-cells in general comes under the general head of thromboplastic substance. It has been designated by the author, for convenience, as thromboplastin and it has been shown⁵ that the essential constituent in this material is a phosphatid which corresponds in its reactions with that member of the group known as kephalin. In shed blood the platelets furnish both thromboplastin and prothrombin. Their disintegration therefore exerts a double influence in hastening the process of clotting; in the first place by furnishing a supply of thromboplastin which neutralizes the antithrombin and thus disturbs the equilibrium existing in the blood between the prothrombin and antithrombin, and secondly by yielding an additional supply of prothrombin which displaces the equilibrium in the same direction. The main object of the present paper is to support the view that calcium salts alone are concerned in the activation of prothrombin to thrombin, and that there is no necessity for assuming the participation of a hypothetical kinase in this process.

THE SEPARATION OF PROTHROMBIN FROM BLOOD-PLASMA

To demonstrate the correctness of the view just stated it is necessary to isolate the prothrombin from the other fibrin factors found in the blood-plasma. The efforts made to effect this separation have been successful in part at least. The method finally adopted was the following: Blood taken from the carotid was oxalated in the usual manner (1 part of a 1 per cent solution of sodium oxalate, made up in 0.9 per cent solution of sodium chloride, to 8 or 9 parts of blood). The mixture was centri-

⁴ Austin and Pepper: *Archives of Internal Medicine*, 1913, xi, 305.

⁵ Howell: *American Journal of Physiology*, 1912, xxxi, 1.

fugalized to obtain a clear plasma. The plasma was sucked off, and, in small portions of 4 to 6 cc., was precipitated by the addition of an equal volume of acetone. The precipitate was thrown at once on a filter and filtration effected quickly by an air-pump. As soon as the liquid had run through, the filter was filled with ether which was also sucked through rapidly. The gelatinous precipitate was spread thin upon the paper and dried rapidly in the current of air from an electric fan. This dried material was kept until needed in a desiccator. The prothrombin was obtained from the dried precipitate by extracting it with water to which a trace of sodium bicarbonate was added. Extraction was made for one-half to one hour and the solution was then filtered. The filtrate was usually opalescent. The presence of prothrombin in this solution was demonstrated easily by the reaction with fibrinogen and calcium solutions, according to the following example:

<i>Fibrinogen</i> <i>drops</i>	<i>CaCl₂ (0.5 per cent)</i> <i>drops</i>	<i>Prothrombin</i> <i>drops</i>	<i>Coagulation Time</i>
10	2		No clot in 24 hours
10		5	No clot in 24 hours
10	2	5	Solid clot in 4 minutes

Occasionally the extract of prothrombin gave a feeble clot after many hours upon the addition of calcium solution, indicating that a small amount of fibrinogen had passed into solution, but in the great majority of the extracts made this reaction did not occur. The only variation from the example cited above lay in the rapidity with which the calcium prothrombin solution caused the fibrinogen to clot. The time necessary for the clotting showed considerable irregularity in the earlier experiments, owing, it is believed, to the tendency of the acetone to render the prothrombin insoluble. In the later experiments in which the acetone was removed as quickly as possible after precipitation, by suction and by washing once with ether, the reaction was much more regular. It is evident that the extracts of the dried precipitate contained a prothrombin material, and for its conversion to active thrombin the presence of a calcium salt was all that was required. There is however one objection to the validity of

this conclusion which must be considered. Assuming that a third factor, a kinase, is concerned in the reaction, and assuming also that this kinase was present in the oxalated plasma, it is possible that it may have been precipitated by the acetone and been redissolved in the extract made from the dried precipitate. As far as the author is aware there is no accepted test for the presence of this kinase other than the existence of thrombin, unless it be admitted that the kinase of Morawitz is identical with thromboplastin, that is, with the substance in tissue-extracts which facilitates or initiates clotting. Tissue extracts, or the kephalin obtained from them, are characterized by their power to cause clotting in peptone-plasmas, and this reaction was used therefore upon the extracts of prothrombin to ascertain whether they contained thromboplastic material. A peptone plasma was chosen which did not clot spontaneously even when diluted with an equal volume of water. Addition of tissue extracts or kephalin solution caused clotting, but the prothrombin extracts were without effect. It may be said therefore that these extracts were lacking in any effective amount of thromboplastic material. In the earlier experiments with the prothrombin extracts it was noted that addition of kephalin solution to the calcium-prothrombin mixture often hastened greatly its action upon fibrinogen. In the later experiments in which the method of quick filtration and washing with ether was used this facilitating action of the kephalin was much reduced or was lacking entirely. From the author's point of view this facilitating effect of kephalin would indicate that some antithrombin was present at times in the solutions of prothrombin, particularly in the earlier experiments. Direct examination of the extracts made in several cases bore out this explanation.

The prothrombin extract was heated first to 60°C. to destroy or weaken the prothrombin and its effect was then tested upon known mixtures of thrombin and fibrinogen in comparison with controls, according to the following schema.

<i>Heated Prothrombin Extract drop</i>	<i>Thrombin drops</i>	<i>Time Interval hour</i>	<i>Fibrinogen drops</i>	<i>Coagulation Time</i>
1	1	1	10	1½ to 2½ hours
1	2	1	10	40 minutes
1	4	1	10	30 minutes
<i>Control with water drop</i>				
1	1	1	10	50 minutes
1	1	1	10	25 minutes
1	1	1	10	20 minutes

Several experiments of this kind gave similar results.

In the method of preparation used for the prothrombin we must believe that more or less of the antithrombin contained in the blood-plasma may appear in the final extract. When the amount of antithrombin present is at all noticeable the use of kephalin facilitates the clotting effect of the calcium prothrombin mixture. In preparations in which the amount of antithrombin is reduced or absent the calcium prothrombin mixture clots the fibrinogen promptly and its effect is increased but little if any by the addition of kephalin solutions.

The prothrombin extracts obtained by the method described were quite stable. When left at ordinary temperatures they maintained their efficiency for long periods, usually in fact until some signs of putrefactive changes were apparent. Heated to 60°C. their activity was markedly diminished but not wholly destroyed. It was found that these prothrombin extracts might be reprecipitated one or more times with acetone, but in each successive precipitate the amount of prothrombin present was reduced, so that the use of this method for the further purification of the prothrombin was abandoned.

THE PRESENCE OF PROTHROMBIN AND THROMBOPLASTIN IN BLOOD-PLATELETS

Occasion was taken during these experiments to verify the reports of other observers regarding the presence of prothrombin and of thromboplastin (Morawitz, Bayne—Jones) in the blood-platelets. The platelets were obtained in the usual way by differential centrifugalization and were washed carefully in a

large volume of saline solution (0.9 per cent sodium chloride). They were then extracted with water to which a trace of sodium bicarbonate was added (5 drops of a half per cent solution to 10 cc. of water). The extract thus made had no coagulating effect at all upon a solution of fibrinogen when used alone, but together with calcium chloride caused clotting in a short time. Example:

Fibrinogen, 10 drops + Plate extract 5 drops gave no clot in 24 hours.

Fibrinogen, 10 drops + Plate extract 5 drops, + CaCl_2 (0.5 per cent) 2 drops gave clot in 20 minutes.

The platelets therefore either contain prothrombin within their cytoplasm or else they carry this material down from the plasma in some sort of adsorptive combination. The first alternative would seem to be much more probable.

The presence of thromboplastin in the platelet extract was demonstrated by its reaction with peptone plasma. Example:

Peptone plasma 10 drops + Platelet extract 10 drops + water 10 drops gave clot in 1 hour.

Peptone plasma 10 drops + water 20 drops gave no clot in 24 hours.

Peptone plasma 10 drops + water 10 drops + prothrombin extract 10 drops gave no clot in 24 hours.

The difference between the platelet extract and the prothrombin extract in regard to thromboplastic substance is brought out clearly in tests of this kind.

THE EFFECT OF BARIUM, STRONTIUM AND MAGNESIUM SALTS UPON PROTHROMBIN

Since by the method described above prothrombin was obtained separated more or less completely from the other fibrin factors, it seemed to be a suitable opportunity to determine whether barium, strontium and magnesium share with calcium the property of activating prothrombin to thrombin. A single experiment upon this point seemed to be sufficiently decisive. One-half per cent solutions were made of calcium chloride, calcium nitrate, strontium chloride, magnesium chloride and barium chloride and their action was tested upon mixtures of fibrinogen and prothrombin extract. The result was as follows:

Fibrinogen 10 drops + Prothrombin extract 5 drops gave no clot in 24 hours.

Fibrinogen 10 drops + Prothrombin extract 5 drops + calcium chloride 2 drops gave clot in 10 minutes.

Fibrinogen 10 drops + Prothrombin extract 5 drops + calcium nitrate 2 drops gave clot in 15 minutes.

Fibrinogen 10 drops + Prothrombin extract 5 drops + strontium chloride 2 drops gave no clot in 6 hours, but partial floating clot between 6 and 20 hours.

Fibrinogen 10 drops + Prothrombin extract 5 drops + magnesium chloride 2 drops gave no clot in 6 hours, but feeble floating clot between 6 and 20 hours.

Fibrinogen 10 drops + Prothrombin extract 5 drops + barium chloride 2 drops gave no clot in 24 hours.

PROTHROMBIN IN THE BLOOD-PLASMA

After the blood is shed the disintegration of the blood-platelets yields no doubt a supply of thromboplastin and prothrombin. Presumably also during the normal circulation these elements undergo dissolution of a more gradual character and we should expect therefore to find both substances among the constant constituents of the circulating blood. To demonstrate that this is actually the case is difficult or impossible with the methods at our disposal at present. By centrifugalization or by filtration through a Berkefeld filter one may remove all corpuscular elements from the blood, but it remains possible of course that some of the platelets may undergo disintegration during this process and thereby furnish to the plasma certain materials not present normally in the circulating blood. As far as the author can see this possible error can not be excluded. One can only use every known precaution to keep the corpuscles intact. Both of the methods suggested above have been attempted with blood-plasma as well as lymph-plasma and the results, as far as they go, indicate the presence of both prothrombin and thromboplastin in the plasma of the circulating blood and lymph, although in the lymph the thromboplastin at least is in much smaller concentration than in the blood. In the experiments with centrifugalized plasma the procedure used was as follows:

The fasting animal was bled from the carotid through a vaseline canula into a paraffined centrifugal tube containing sufficient sodium oxalate (made up in 0.9 per cent solution of sodium chloride) to give a final mixture of 0.1 per cent sodium oxalate. Only the first sample (40 cc.) collected was used. As soon as the tube was filled the contents were mixed by pouring carefully into a second paraffined centrifugal tube. This latter tube was then centrifugalized at half speed (1500 to 2000 revolutions) for 20 minutes. The supernatant plasma was pipetted off through a vaselined tube, a portion was kept for examination and the remainder was centrifugalized for one hour at full speed (3000 revolutions). Two specimens of plasma were thus obtained, one completely centrifugalized, very clear and presumably free from all formed elements; the other incompletely centrifugalized and containing still many platelets in suspension. When these two specimens were recalcified, if the expression may be used, by the addition of optimum amounts of calcium chloride, it was found that the completely centrifugalized plasma required a longer time to clot. The difference however was not very great. In the most successful experiment the incompletely centrifugalized plasma clotted in 10 minutes, the other in 30 to 35 minutes. In lymph a much greater difference was observed in most cases. The following records were obtained:

	<i>Incompletely centrifugalized</i>	<i>Completely Centrifugalized</i>
March 3, 1914	13 minutes	No clot in 3 hours
March 8, 1914	30 "	Clot between 2 and 20 hours
March 17, 1914	15 "	20 to 25 minutes
March 21, 1914		No clot in 20 hours
March 24, 1914	30 to 40 "	Between 5 and 20 hours
March 27, 1914	25 "	Between 3 and 15 hours

The results with lymph-plasma were variable, but in general the complete centrifugalization greatly prolonged the time of clotting after the addition of calcium. That this delay was due to a deficiency of thromboplastin rather than of prothrombin was indicated by the fact that the addition of a kephalin-solution (tissue extract) to the completely centrifugalized plasma, together with the calcium, was followed by prompt clotting within a

few minutes (4 to 30). The experiments upon blood-plasma passed through a Berkefeld filter are described by Goddard in a separate paper⁶ and may be referred to very briefly. Using all possible precautions in regard to vaselining or paraffining the tubes it was found that the first portion of the filtrate collected might fail to clot even with thrombin, indicating therefore that the fibrinogen had been strained off. In intermediate portions the addition of calcium alone might be insufficient to cause clotting while calcium plus tissue-extract (kephalin) was effective. In these portions therefore prothrombin was present while thromboplastin was lacking or was present in ineffective amounts. In the last portions of the filtrate calcium chloride alone caused clotting as in the unfiltered plasma, although the time required was usually much longer. As far as these experiments go therefore they support the conclusion that prothrombin and thromboplastin, in variable amounts, are constantly present in the circulating plasma.

SUMMARY

1. Prothrombin may be precipitated by acetone from oxalated blood-plasma and be obtained in solution free from the other fibrin factors, with the possible exception of traces of antithrombin.
2. The prothrombin is converted to active thrombin in the presence of calcium salts. No third factor seems to be necessary for this activating process.
3. The activating property of calcium salts is exhibited to a much less extent by strontium salts, and very feebly by magnesium salts.
4. The prothrombin in salt free or salt poor solutions is weakened but not destroyed by heating to 60°C.
5. Aqueous extracts of the blood-platelets show the presence of both prothrombin and thromboplastin.
6. Prothrombin (probably some thromboplastin also) is contained in the plasma of circulating blood. In shed blood disintegration of the platelets furnishes an additional supply of prothrombin and thromboplastin.

⁶ Goddard: American Journal of Physiology, 1914, xxxv.

